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ABSTRACT

Partitioning of copper among copper-binding proteins was evaluated in digestive glands of Mytilus edulis exposed to soluble copper. Groups of mussels were held in flow-through bioassay systems and exposed to 25 µg Cu/L for up to 21 wk. At 3-wk intervals, groups of 25 mussels were removed and the digestive glands were analyzed for copper-binding proteins by gel-permeation chromatography and atomic absorption spectrometry.

Chronic exposure to copper resulted in increased amounts of copper in the low molecular-weight (LMW) protein fraction, which contains metallothioneins, and in the high molecular-weight (HMW) protein fraction, which contains metalloenzymes. Concentrations of copper in the LMW protein fraction increased and then appeared to plateau with long exposure times, whereas those in the HMW protein fraction continued to increase with exposure time.

INTRODUCTION

Adverse effects on mussels of increased Cu concentrations in seawater are well documented (Eisler, 1979). On the other hand, mussels containing high Cu concentrations in tissues have been identified in field studies (Goldberg, 1980). Tolerance of mussels to increased tissue Cu concentration may be related to the presence of detoxifying mechanisms. A process that may be important in metal detoxification in mussels is the binding of metals to low molecular weight (LMW) proteins similar to metallothionein (MT) (Viarengo et al., 1983a; 1984). It is proposed that metals bound to MTs may be less available for competitive binding to enzymes and considered to be detoxified.

Intracellular binding of Cu to MTs has been demonstrated in both field (Talbot & Magee, 1978; Harrison et al., 1983) and laboratory experiments (Viarengo et al., 1980; Viarengo et al., 1981a; Harrison et al., 1983). Also, Viarengo et al. (1980) reported that short-term exposure to Cu results in the induction of Cu MTs in the digestive gland. However, the changes in concentration of Cu in the MTs of the digestive gland upon long-term exposure of mussels to Cu are not known. This study was initiated to determine the changes in partitioning of Cu among Cu-binding proteins in the digestive gland of Mytilus edulis exposed chronically to a soluble Cu concentration that might occur in Cu-contaminated environments.

MATERIALS AND METHODS

Collection and exposure of mussels

Specimens of Mytilus edulis (shell length, 50 to 80 mm) were collected in 1981 from Tomales Bay, California, an area known to be low in environmental pollutants (Risebrough et al., 1980). Mussels used in the first experiment

were collected in the summer and those in the second experiment were collected in the fall. Upon arrival at Lawrence Livermore National Laboratory, Livermore, California, mussels were cleaned of all attached biota and acclimated for 3 d in flowing, aerated seawater at 13°C and 30 ± 1 °/oo salinity. This seawater was low in dissolved organic carbon and metals and had a low Cu complexing capacity (Knezovich et al., 1981).

Groups of 50 randomly selected mussels were placed in 20-L exposure chambers containing 25 µg Cu/L seawater; the flow rate of seawater was 100 ± 5 mL/min. Control mussels were treated similarly to Cu-exposed mussels except that no Cu was added to the seawater.

Replacement of 90% of the seawater in the chambers required approximately 7 h (Sprague, 1969). Continuous aeration of the water in the bioassay chambers ensured thorough mixing. Before the experiment was initiated, all mussels were acclimated for 3 d to the decreased salinity (29 ± 1 °/oo) expected from the dilution of seawater by addition of the stock solutions. All animals were checked three times each week, and any gaping animal not responding to a probe was considered dead and removed. Flow rates were measured twice weekly, and the expected Cu concentrations in the exposure chambers were calculated from the measured flow rates. Calculated concentrations were verified by analysis of the bioassay water. The control seawater contained about 1 µg Cu/L and had a pH of 7.7 ± 0.1 and a temperature of 13 ± 1°C.

The mussels were fed a starch solution (1 mg/L in the exposure chamber) continuously during the study to avoid depletion of the reserves of the digestive gland, which have been shown to be utilized during periods of starvation (Thompson et al., 1974). In addition, 1-L suspension of 1 g powdered, freeze-dried Macrocystis pyrifera was added daily to each chamber.

Groups of 25 animals were sampled at 3-wk intervals. Two separate experiments were performed. The duration of the first experiment was 12 wk, and that of the second was 21 wk. All feeding was terminated 24 h before the animals were sacrificed to allow for purging of the gut (Goldberg, 1980).

The digestive glands were quickly dissected out first and then immediately frozen on dry ice and preserved in a -70°C freezer before homogenization. All samples of digestive glands were homogenized on ice with a Polytron in 2 vol/wt of nitrogen-saturated buffer containing 10% sucrose, 200 K.I.U. Aprotinin (Trasylol, a peptide with a molecular weight of 6000 to 7000 daltons), and 1% 2-mercaptoethanol. An aliquot of each homogenate was reserved for metal analysis. Metal analyses were performed also on digestive gland, kidney, and gill tissue of control mussels used in the second experiment. Five groups of each tissue pooled from five mussels were analyzed.

Isolation and purification of MTs

Homogenates were centrifuged first at 5,000 X g for 15 min at 4°C. The resultant supernatants were centrifuged further at 100,000 X g for 120 min at 4°C. The final clear supernatants (S-100s) were filtered through sterile, 0.45-µm (pore size) filters and were then either chromatographed directly or frozen immediately with liquid nitrogen and stored at -70°C. Aliquots of each S-100 and all pellets were reserved for metal analyses.

Ten ml of each S-100 of the digestive glands from mussels exposed for 12 wk (Experiment 1) were processed on a calibrated Sephadex G-75 column (2.6 X 60 cm) equilibrated with 50mM NH_4HCO_3 (pH 8.6) at 3°C. The flow rate was 25 mL/h. Column eluates were monitored continuously for absorbance at 280 and 254 nm with an Altex Dual Wavelength UV-Detector. Three mL fractions were collected and analyzed for Cu, zinc (Zn), and cadmium (Cd).

One mL of each S-100 of digestive glands from mussels exposed for 21 wk (Experiment 2) were applied to a Waters High-Performance Liquid Chromatograph (HPLC) fitted with a calibrated Varian TSK SW 3000 Gel-Permeation Column (22 X 300 mm). The mobile phase consisted of 50mM Tris-HCl (pH 7.6; 22°C) at 0.5 mL/min. Fractions were collected every minute for 40 min and analyzed for Cu, Zn, and Cd. Molecular absorbance at 280 nm was monitored continuously.

Metal analysis

Total Cu concentrations in seawater from the exposure chambers were determined by atomic absorption spectrometry (AAS) using the method of additions. Column fractions were analyzed directly by flame or graphite furnace AAS.

Tissues for metal analysis by AAS were dried at 100°C, ashed at 450°C, dissolved in a mixture of concentrated HCl and HNO_3 (3:1), and brought to final volume with double-distilled water. A standard reference material of oyster tissue obtained from the National Bureau of Standards was analyzed with the mussel tissues to validate analytical procedures.

RESULTS

Metals in unexposed mussels: Experiments 1 & 2

Cu and Zn concentrations in digestive gland, gill, and kidney tissues for unexposed mussels from both experiments 1 and 2 are shown in Table 1. Concentrations of both metals were considerably higher in mussels for experiment 2 in relation to experiment 1. Additionally, the relative distribution of Zn in different tissues of the two groups differed in that

this metal was concentrated in digestive gland of experiment 1 mussels and in kidney of experiment 2 mussels. These differences in the status of metals in the two experimental groups most likely reflected differences in either biological conditions of the mussels or the environment, and were reflected in differences in responses of the two groups to Cu exposure in the laboratory.

Experiment 1

Cu and UV-absorbance profiles characteristic of all Sephadex G-75 chromatograms are shown in Fig. 1. The total amounts of Cu in the LMW (5,000 to 40,000 dalton) and HMW (>40,000 dalton) fractions were calculated for comparison to the amounts in the pellet and supernatant fluid (Fig. 2). Control mussels sampled at 3, 6, and 12 wk had mean concentrations of Cu in the HMW fraction of 1.9 $\pm$ 1.0, in the LMW fraction of 24 $\pm$ 10, in the supernatant fluid of 20 $\pm$ 11, and in the pellet of 2 $\pm$ 1 µmoles/kg wet tissue.

In mussels exposed to Cu for 3 wk, the amounts of Cu eluted in the four compartments were similar to those in control (unexposed to Cu) mussels. In mussels exposed to Cu for 6 wk, the amount of Cu eluted in the LMW fraction was about 5 times greater than that in the control mussels and in those exposed to Cu for 3 wk. Also, the increase in the LMW fraction was greater than that in the HMW fraction. In mussels exposed to Cu for both 9 and 12 wk, the amount of Cu in the HMW and LMW fractions was slightly greater than that from these same fractions at earlier exposure times. Cu in the HMW fraction appeared to increase continuously, whereas that in the LMW fraction appeared to plateau. Cu levels in pellets obtained from centrifugation of homogenized digestive glands were intermediate to that in the HMW and LMW fractions. In the supernatant fluid, the LMW fraction accounted for a greater proportion of the Cu at early sampling times than at later times.

Elution profiles and concentrations of Zn and Cd in the supernatant fluids of digestive glands (not shown) were similar in both the control and Cu-exposed mussels. In all cases more Zn was present in the HMW fraction than in the LMW fraction whereas the opposite was true of Cd. The mean values for Zn in the HMW and LMW fractions from control mussels were 57 $\pm$ 8 and 42 $\pm$ 11 µmoles/kg wet tissue and for Cd were 1.8 $\pm$ 0.5 and 6.7 $\pm$ 3.0 µmoles/kg wet tissue, respectively.

Experiment 2

Representative Cu and UV-absorbance profiles characteristic of gel permeation HPLC chromatography are shown in Fig. 3. More protein peaks are seen in the profiles obtained by using HPLC than in those obtained by using Sephadex G-75 (cf. Fig. 1 and 3). The Cu profiles obtained by both techniques were similar, however.

In contrast to the results of the first experiment, increases in Cu in the different subcellular compartments were evident at the first sampling period in experiment 2. A lag period preceding the onset of Cu accumulation was not observed. Additionally, the Cu concentration in the pellet of experiment 2 digestive glands was greater than that of experiment 1 (cf. Fig. 2 and 4). In other respects, general patterns of Cu accumulation were similar in the two experiments. The order of concentration of Cu in the various compartments over time was supernatant fluid >LMW fraction >pellet >HMW fraction in experiment 2; Cu in the LMW fraction again appeared to plateau at the later time periods, while that in the HMW fraction continuously increased.

DISCUSSION

The distribution of metals among metal-binding proteins in mussels exposed to increased metal concentrations has been determined in a number of tissues (Noel-Lambot, 1976; Viarengo et al., 1980; Viarengo et al., 1981a; Roesijadi and Hall, 1981; Roesijadi, 1982). However, in most experiments, the duration of exposure was short and the mussel population was sampled only once. Because mussels are subjected to chronic exposure to low levels of metals in many ecosystems, data on the response of mussels to chronic exposure are needed to provide information on the rate of loading of metals on metal-binding proteins and on the capacity of the putative metal-binding protein detoxification system.

To understand the causes of metal toxicity, we must attempt to elucidate the cellular and biochemical processes involved in the metabolism of metals. Our results, together with other data (George and Viarengo, in press), indicate that the complexing of metals with MTs is an important process related to metal sequestration in Mytilus edulis. In our experiments, the quantities of Cu associated with the LMW protein fraction, which includes MTs, increased with exposure time. However, the shape of the curves indicated that the amount of Cu associated with MTs appeared to plateau. Changes with time in the amount of metal associated with LMW proteins were determined also by Roesijadi (1982), who followed the amount and distribution of mercury accumulated by mussel gill tissue during a 28-d period. He reported that there was apparent saturation of the mercury-binding proteins during the later portion of the time course. Limitation in the uptake of Cu could result because the quantities of MTs that can be synthesized are controlled genetically or because the increased quantities of Cu in the cytosol interfere with the synthesis of MTs or with some other critical metabolic process.

Concomitant with the increase of Cu in the LMW protein fraction, Cu associated with the HMW fraction in Mytilus edulis continuously increased with time. The HMW fraction includes Cu metalloenzyme and other metalloenzymes required for metabolic activity. These may be inhibited by nonspecific binding of excess cellular Cu. Binding of Cu to MTs, which can compete with other cellular ligands for Cu, may have reduced the rate of incorporation of Cu into the metalloenzyme pool and reduced the possibility of inhibition by Cu.

In the population of mussels used in experiment 1, we found that some Cu was associated with LMW proteins in the cytosol of digestive glands of mussels that were not exposed to Cu, but that no large increases occurred in the LMW protein fraction until after 3 wk of exposure to 25 µg Cu/L. In the population of mussels used in experiment 2, we found that the quantities of Cu associated with the LMW fraction increased during the first 3 wk of exposure. The difference in the time before there was an increase in Cu associated with MTs may reflect differences in the exposure history of the populations used in the two experiments. Although the mussels were obtained from the same site, they were collected at different times. In mussels from experiment 2, prior induction of MTs in the digestive gland may have occurred in the field. This was indicated by the high metal content of the mussels before exposure to Cu in the laboratory. If prior induction had occurred, additional MT may have been immediately available for binding Cu, possibly through displacement of Zn, because the affinity of Cu to MT is greater than that of Zn. Alternatively, these mussels may have been pre-disposed to faster and higher levels of induction as a result of their field exposure. In mussels from experiment 1, the absence of an increase in Cu associated with MTs in

unexposed mussels and those exposed to Cu for 3 wk probably indicated that induction had not occurred prior to Cu exposure in the laboratory. These mussels also had a lower fraction of Cu associated with the pellet of the digestive gland both before and after exposure to Cu for equivalent times. Because a significant fraction of the metals in the pellet may be associated with lysosomes and residual bodies (Viarengo et al., 1981b), changes in the pellet may reflect detoxification of metals by membrane-bound vesicles (George et al., 1982). Viarengo et al., (1981b) demonstrated that Cu continued to increase in the lysosome fraction after exposure to Cu was terminated. Viarengo et al., (1983b) presented data indicating that Cu-thioneins accumulate in lysosomes and residual bodies and stated that this process may play an important role in the homeostasis and detoxification of Cu.

Relationships among possible detoxification processes in a single tissue and among detoxification processes in different tissues are poorly understood. Further experimentation is required before interactions and relationships are defined.

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TABLE 1

METAL CONCENTRATION (µmoles/kg WET TISSUE) IN TISSUES POOLED FROM UNEXPOSED *MYTILUS EDULIS*

Tissue	Copper	Zinc
	Experiment 1[a]	
Digestive gland	46	240
Gill	10	71
Kidney	34	81
	Experiment 2[b]	
Digestive gland	82 ± 31	400 ± 110
Gill	45 ± 17	210 ± 80
Kidney	110 ± 47	4500 ± 1500

[a] One group of each tissue pooled from 25 mussels.

[b] Five groups of each tissue pooled from 5 mussels.

FIGURE LEGENDS

1. Copper elution profile and UV-absorbance profile of 100,000 X g supernatant fluid (S-100) of digestive glands pooled from 25 Mytilus edulis exposed to 25 µg Cu/L for 12 wk (Experiment 1). Separation of proteins in the S-100 was performed using a Sephadex G-75 column.

2. Changes with time in the quantities of Cu associated with the supernatant fluid, pellet, HMW protein fraction, and LMW protein fraction of digestive gland homogenates (Experiment 1). Means of values from control mussels sampled at 3, 6, and 12 wk are shown at y intercept.

3. Copper elution profile and UV-absorbance profile of 100,000 x g supernatant fluid (S-100) of digestive glands pooled from 25 Mytilus edulis exposed to 25 µg Cu/L for 21 wk (Experiment 2). Separation of proteins in the S-100 was performed using a high-performance liquid chromotograph fitted with a Varian TSK SW 3000 gel-permeation column.

4. Changes with time in the quantities of Cu associated with the supernatant fluid, pellet, HMW protein fraction, and LMW protein fraction of digestive gland homogenates (Experiment 2).

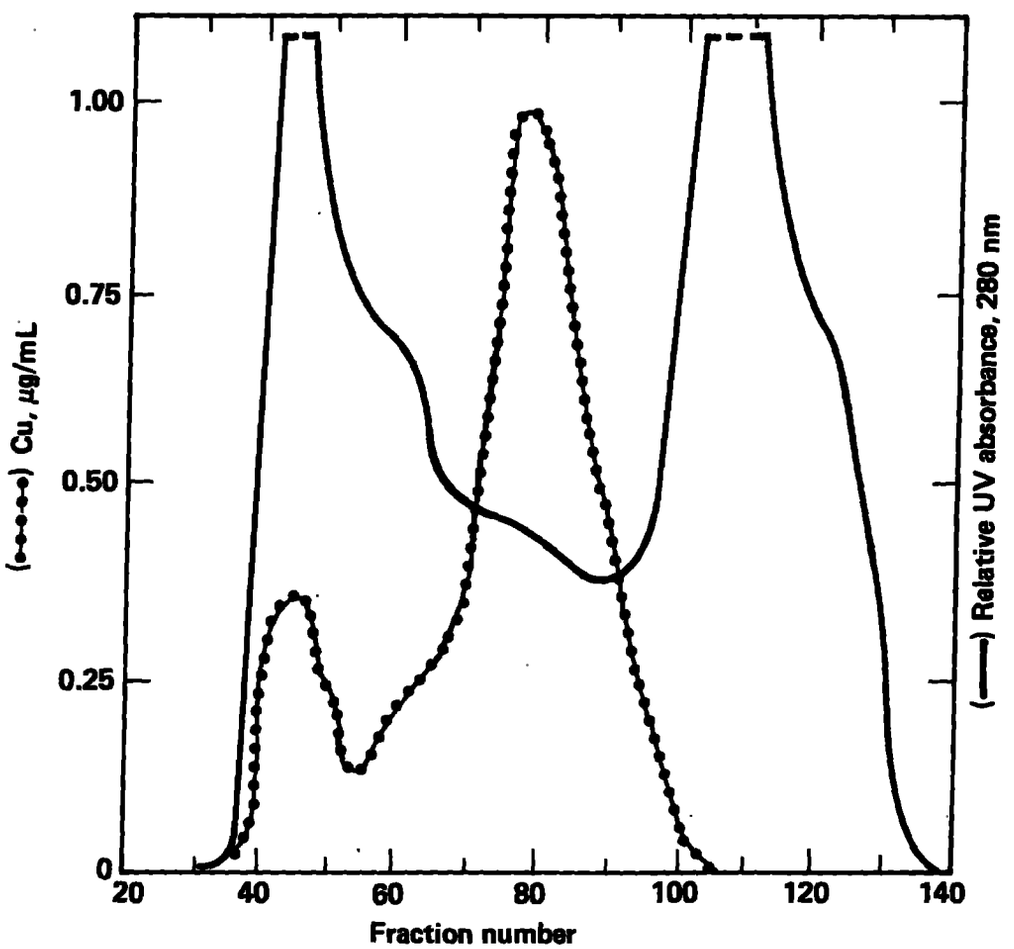
1.00
0.75
0.50
0.25
0
20
40
60
80
100
120
140
Fraction number
(•-•-•) Cu, µg/mL
(——) Relative UV absorbance, 280 nm

Figure 1

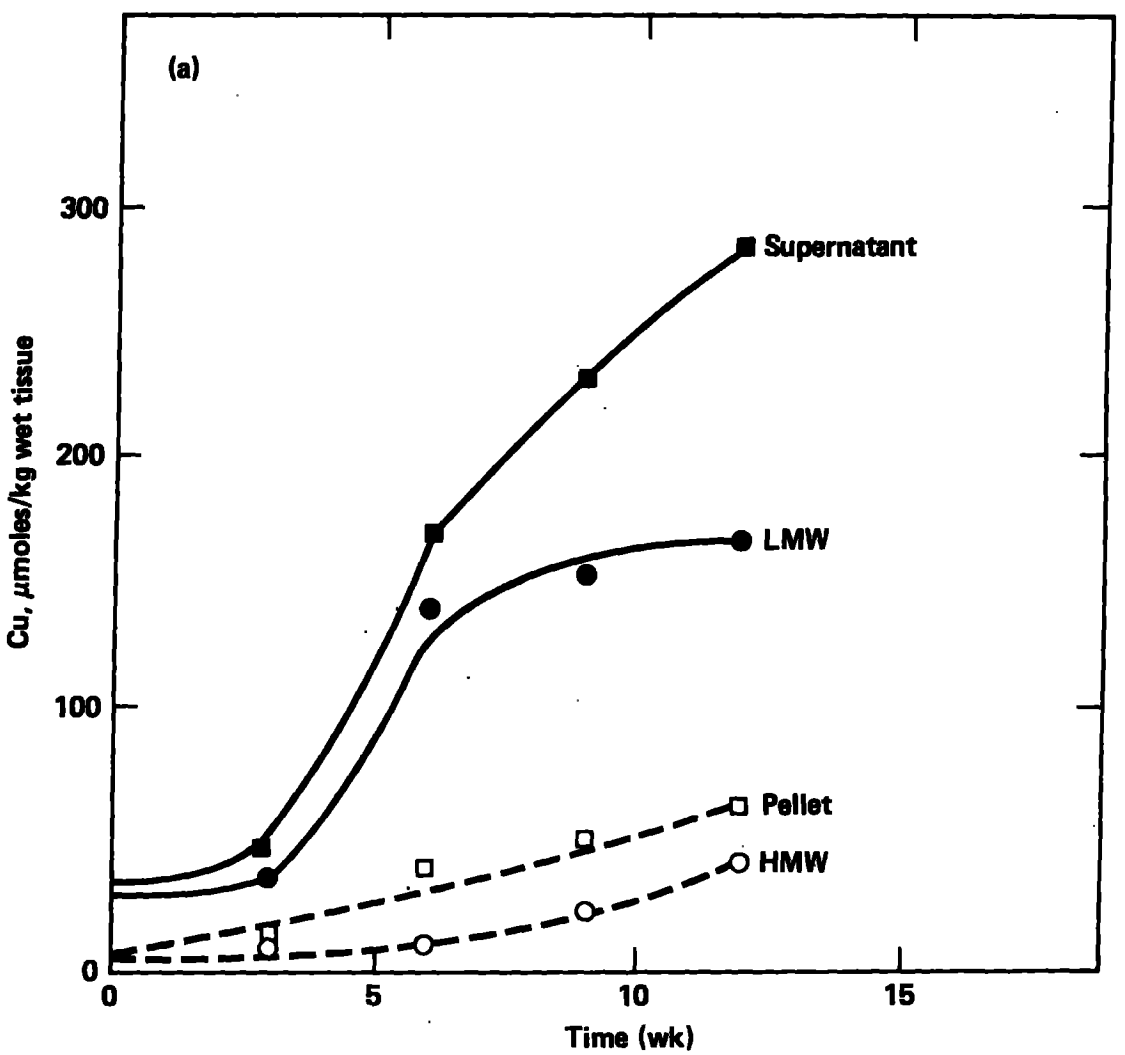
(a)
300
200
100
0
Cu, μmoles/kg wet tissue
Supernatant
LMW
Pellet
HMW
0
5
10
15
Time (wk)

Figure 2

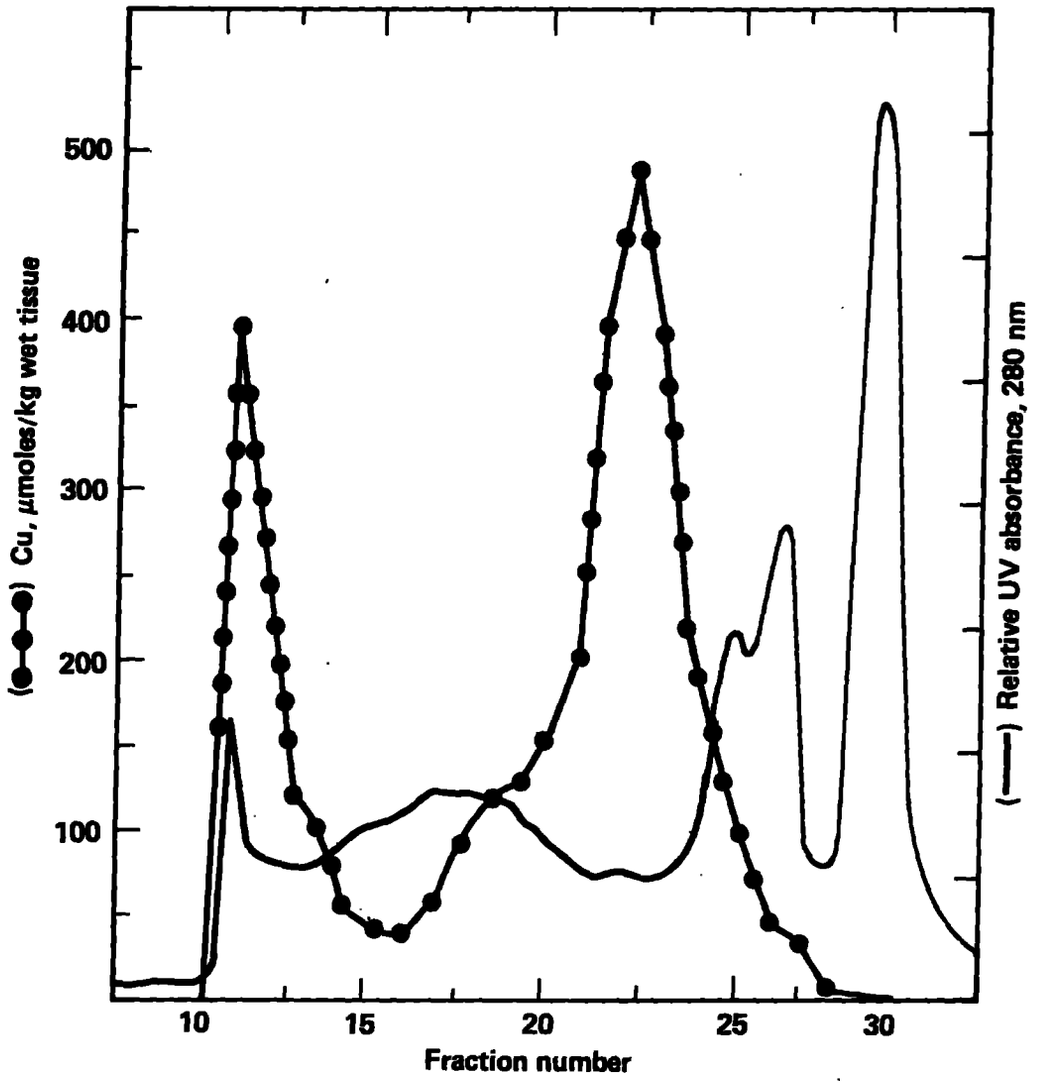
500
400
300
200
100
(●—●) Cu, μmoles/kg wet tissue
(——) Relative UV absorbance, 280 nm
10
15
20
25
30
Fraction number

Figure 3

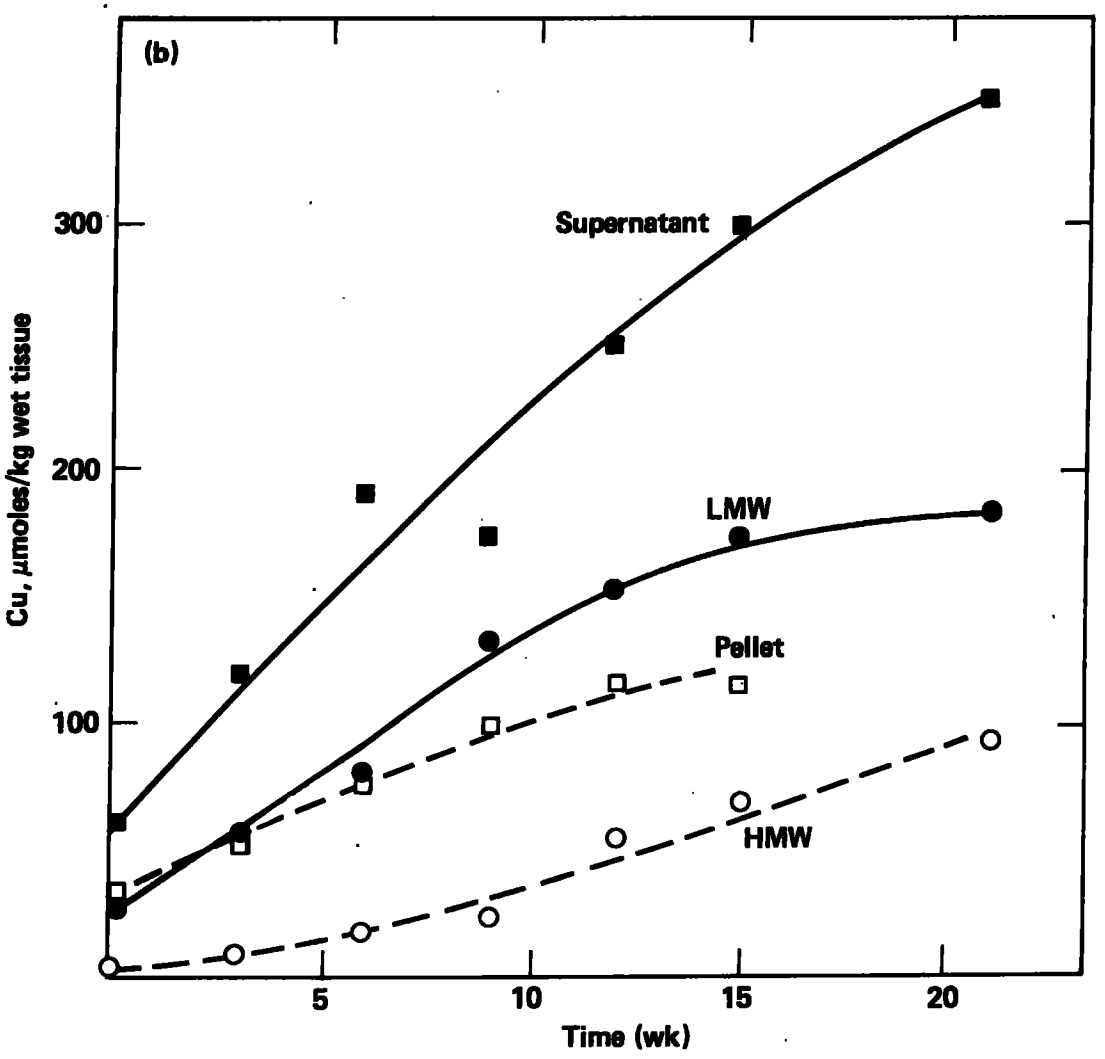
(b)
300
200
100
Cu, μmoles/kg wet tissue
Supernatant
LMW
Pellet
HMW
5
10
15
20
Time (wk)

Figure 4